

10/PRTS

## SPECIFICATION

## MAMMALIAN GENES INVOLVED IN CIRCADIAN PERIODS

5 Technical Field

The present invention relates to mammalian genes whose expression changes with a circadian period.

Background Art

10 Many biochemical processes, physiological processes, and behavioral processes in various organisms ranging from microorganisms to vertebrates exhibit circadian rhythms (Edmunds, L. N. J., Cellular and Molecular Basis of Biological Clock, Springer-Verlag, New York, 1988). Several genes have been  
15 suggested to be involved in circadian rhythms.

For example, two mammalian circadian clock mutations have been confirmed thus far. They are Clock of the mouse (Vitaterna, M. H., et al., Science 264: 719-725, 1994) and tau of the hamster (Ralph, M. R. and Menaker, M., Science 241: 1225-1227, 1988). The Clock  
20 gene has recently been identified and is believed to encode a transcription factor in the circadian clock (Moor, R. Y. and Eichler, V. B., Brain Res. 42: 201-206: 1972; Stephan, F. K. and Zucker, I., Proc. Natl. Acad. Sci. USA 69: 1583-1586, 1972). On the other hand, the tau gene has not yet been cloned.

25 The period (per) gene has been isolated from *Drosophila* as a gene necessary for the expression of circadian rhythms for locomotive activities and eclosion behavior (Konopka, R. J. and Benzer, S., Proc. Natl. Acad. Sci. USA 68: 2112-2116, 1971). In the brain of the fly the oscillation of the levels of the per mRNA  
30 and of the PERIOD (dPER) protein are thought to determine the rhythms (Hardin, P. E., et al., Nature 343: 536-540, 1990; Zerr, D. M., et al., J. Neurosci. 10: 2749-2762, 1990). However, per homologues in other organisms than insects have not been identified.

35 Disclosure of the Invention

An object of the present invention is to provide novel

mammalian proteins and the genes thereof that are involved in the circadian period. More specifically, the object is to provide mammalian proteins and the genes thereof that are functionally equivalent to those of the *Drosophila* period (per) gene product.

5 To attain the above object, the present inventors focused on a region expected to play a functionally important role within the *Drosophila* gene known to be involved in the circadian rhythms, and performed a type of PCR, which had been developed on our own, using the primers designed based on the sequence of the region. As a result,  
10 we succeeded in isolating a human gene that corresponds to the above-mentioned *Drosophila* gene. We also succeeded in isolating a mouse gene that corresponds to the human gene by using the isolated human gene as a probe. Furthermore, we analyzed structures of the proteins encoded by the human and the mouse genes thus isolated and  
15 discovered that these proteins highly conserve the functional domains and the structural domains that have been identified in the *Drosophila* protein. In addition, analysis of the expression of the isolated mouse gene in the suprachiasmatic nucleus, which is the region responsible for functioning as a circadian pacemaker in the  
20 mammalian brain, revealed that the expression of the gene fluctuates with a circadian period.

Namely, the present invention relates to proteins and the genes thereof that are involved in the circadian periods of mammals, and more specifically to

- 25 (1) a protein derived from a mammal whose expression level in the suprachiasmatic nucleus (SCN) fluctuates with a circadian period,  
(2) a protein of (1) wherein the mammal is a human,  
(3) a protein of (1) wherein the mammal is a mouse,  
(4) a protein involved in the formation of circadian rhythm in  
30 the suprachiasmatic nucleus (SCN) comprising the amino acid sequence described in SEQ ID NO: 1 or said sequence in which one or more amino acids are substituted, deleted, or added,  
(5) a protein involved in the formation of circadian rhythm in  
the suprachiasmatic nucleus (SCN) comprising the amino acid  
35 sequence described in SEQ ID NO: 2 or said sequence in which one or more amino acids are substituted, deleted, or added,

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homologous with that of the *Drosophila* protein (the period gene product) (Citri, Y., et al., Nature 326: 42-47, 1987). The period gene is required for the expression of the circadian rhythms of locomotive activities and hatching behavior in *Drosophila* (Konopka, R. J. and Benzer, S., Proc. Natl. Acad. Sci. USA 68: 2112-2116, 1971). The oscillations of its mRNA and protein levels in the fly brain are thought to determine the rhythms (Hardin, P. E., et al., Nature 343: 536-540, 1990; Zerr, D. M., et al., J. Neurosci. 10: 2749-2762, 1990). These two proteins show highly homologous with the *Drosophila* protein in the PAS domains which have been suggested to be structurally and functionally important based on the genetic and biochemical studies (Baylies, M. K. et al., Nature 326: 390-392, 1987; Saez, L. and Young, M. W., Neuron 17: 911-920, 1996).

Recently King et al. have cloned the mammalian "Clock" gene, which encodes a bHLH-PAS-polyQ polypeptide (King, D. P., et al., Cell 89: 641-653, 1997; Antoch, M. P., et al., Cell 89: 655-667, 1997). The proteins of the present invention can form dimers with other molecules such as "CLOCK" by means of the PAS-PAS interaction in the circadian clock system.

The proteins of the present invention can be prepared as a recombinant protein utilizing the genetic recombinant technology, or as a natural protein. A recombinant protein can be prepared by culturing the cells transformed with DNA encoding the protein of the present invention as described later. A natural protein can be isolated, for example, from the somatic cell tissues, such as brain, pancreas, kidney, skeletal muscle, liver, lung, placenta, heart, spleen, and testis using an affinity column with an appropriate carrier bound to an antibody that is prepared using the above-mentioned recombinant protein of the present invention.

It is possible for a person skilled in the art to prepare a protein substantially identical to the protein described in SEQ ID NO: 1 or SEQ ID NO: 2 by making amino acid substitutions and other modifications to the protein described in SEQ ID NO: 1 using known methods. Mutations of amino acids in a protein may also occur spontaneously. Thus, the present invention includes modified proteins that result from the modification of amino acids of the

protein described in SEQ ID NO: 1 or 2 by substitution, deletion, or addition, and are involved in the formation of circadian rhythms in the suprachiasmatic nucleus (SCN). The known methods to modify amino acids include the ODA (Oligonucleotide-directed Dual  
5 Amber)-LA PCR method (Hashimoto-Gotoh, T., et al., Gene 152: 271-275, 1995). The amino acids to be substituted are usually within 10 amino acids, preferably within 6 amino acids, and more preferably within 3 amino acids.

It is routine for one skilled in the art to obtain proteins  
10 that are substantially functionally equivalent to the protein described in SEQ ID NO: 1 or 2 from DNAs that are highly homologous with the DNA having a sequence described in SEQ ID NO: 3 or 4 and isolated from other organisms using such methods as the known hybridization technique (Church, G. M. and Gilbert, W., Proc. Natl.  
15 Acad. Sci. USA 81: 1991-1995, 1984; Sambrook, J., et al., Molecular Cloning, 2<sup>nd</sup> ed., 1989) based on the DNA sequence described in SEQ ID NO: 3 or 4 (or part thereof). Thus the proteins encoded by the DNA that hybridizes with the DNA sequence described in SEQ ID NO: 3 or 4, which are involved in the formation of circadian rhythms  
20 in the suprachiasmatic nucleus (SCN), are also included in the proteins of the present invention. The source of the DNA for hybridization includes mammals such as rats, dogs, cats, monkeys, whales, cattle, pigs, and horses. The DNA encoding the proteins from these other organisms should usually highly homologous with  
25 the DNA described in SEQ ID NO: 3 or 4. "Being highly homologous" means having at least 60%, preferably at least 70%, more preferably at least 80%, and still more preferably at least 90% of sequence identity with the DNA described in SEQ ID NO: 3 or 4. The hybridization for isolating such DNAs can be performed, for example,  
30 in a mixture consisting of 6 x SSPE, 5 x Denhardt's solution, 0.5% SDS, 100 µl/ml denatured salmon sperm DNA, and 50% formamide, usually at 42°C, less stringently at 32°C, or more stringently at 65°C.

The present invention also relates to DNAs encoding the proteins of the present invention described above. The DNAs  
35 encoding the proteins of the present invention can be cDNA, genomic DNA, or synthetic DNA. The DNAs of the present invention can be

utilized, for example, to manufacture the proteins of the present invention as recombinant proteins. Namely, the DNA encoding a protein of the present invention (for example, the DNA described in SEQ ID NO: 3 or 4) is inserted into an appropriate expression vector, appropriate cells are transformed with the vector, the transformants are cultured, and the expressed protein is purified to prepare the proteins of the present invention as recombinant proteins.

The preferred cells used for the production of the recombinant proteins include E. coli, yeast, insect cells, and animal cells. The vectors used to express the recombinant proteins within these cells include the pET system, pAUR system, baculovirus vectors (pBlue Bac, etc.), and the CMV or RSV promoter-driven vectors, etc.

The transfection of the vector into the host cell can be done, for example, by electroporation for E. coli and yeast, and the liposome method for insect cells and animal cells. The lithium acetate method can also be used for yeast.

The recombinant protein can be purified from the transformant, for example, by ion exchange, gel filtration, or anti-Per antibody column chromatography.

The proteins or the DNAs of the present invention are applicable to treat disorders related to circadian rhythms, such as sleep phase delay syndrome, sleep phase progression syndrome, non-circadian sleep-wake syndrome, irregular sleep-wake disorder, and time difference syndrome (so-called jet lag). They are also applicable to the labor and health management of irregular night time workers and to prevention of night poriomania in dementia.

#### Brief Description of the Drawings

Figure 1 shows the amino acid sequences within the PAS repeats (arrows) that were used to design the primers for IMS-PCR.

Figure 2 is a photograph showing an electrophoresis image of 3 bp ladder markers that were electrophoresed on a 10% non-denaturing PAGE gel in a non-continuous buffer solution system. A 10 bp DNA ladder (BRL) was electrophoresed on lane M.

Figure 3 is a photograph showing an electrophoresis image of

the IMS-PCR product (lanes marked with arrows) that was electrophoresed along with 59 bp, 65 bp, and 68 bp of the 3 bp ladder markers (lanes marked with asterisks).

Figure 4 shows an amino acid sequence comparison among the PERIOD family members. hDIAL, mDIAL, and PERIOD indicate the human, the mouse, and the *Drosophila* version of PERIOD, respectively. Shaded or dotted boxes indicate homologous sequences, and C1 through C6 indicate regions conserved among different *Drosophila* species.

Figure 5 shows an amino acid sequence comparison among the PERIOD family members. hDIAL, mDIAL, and PERIOD indicate the human, the mouse, and the *Drosophila* version of PERIOD, respectively. Shaded or dotted portions indicate homologous sequences. Sequences corresponding to NLS, the PAS-A repeats, the PAS-B repeats, and CLD are underlined, and the TG repeats (the SG repeats in the human and mouse PER) are boxed. Amino acid identities between the human PERIOD and the mouse PERIOD are indicated by asterisks above the human PERIOD sequence. The identities and homologies between the mammalian PERIOD and the *Drosophila* PERIOD are indicated by asterisks and open circles below the *Drosophila* PERIOD sequence.

Figure 6 is a photograph showing the northern blot analysis of hPER. hPER was bound to the filter as a probe, and then G3PDH was bound as a loading control.

Figure 7 is a photograph showing the northern blot analysis of mPer. mPer was bound to the filter as a probe, and then G3PDH was bound as a loading control.

Figure 8 is a photograph showing the results of *in situ* hybridization of mPer in the mouse brain under the LD (top) and the DD (bottom) conditions. SCN is indicated by arrows. The bar indicates 2 mm.

Figure 9 shows the results of quantification of *in situ* hybridization data under the LD (top) and the DD (bottom) conditions. Each data point is the average  $\pm$  SEM (n=5). \*\* indicates significance at the 1% significance level, and \* at the 5% significance level, compared with the values at ZT16 and CT16. The white portion of the bar represents the light period, and the black portions the dark periods.

Figure 10 shows the results of the competitive RT-PCR analysis on the mPer mRNA under the LD (top) and the DD (bottom) conditions. ΔmPer indicates a competitive factor for mPer and Δβ-actin indicates a competitive factor for β-actin. The white portion of the bar represents the light period, and the black portions the dark periods.

#### Best Mode for Carrying out the Invention

The present invention is illustrated in detail below with reference to the following examples, but is not to be construed as being limited thereto.

#### Example 1 Isolation of the mammalian homologues of per

In order to isolate the mammalian homologues of per, the inventors have developed a novel method, intramodule scanning (IMS) -PCR. The principle of the method is based on the fact that in the human genome short stretches of DNA sequences (modules) that encode short polypeptide fragments (motifs) are scattered over long genomic distances. If a sufficient number of "intramodule scanning" primers are used to cover the entire length of a gene, the module can be screened with equal frequencies irrespective of their expression levels.

Genetic and biochemical studies have suggested that the PAS domains in dPER are structurally and functionally important (Baylies, M. K. et al., Nature 326: 390-392, 1987; Saez, L. and Young, M. W., Neuron 17: 911-920, 1996). Therefore, we designed 18 different primers corresponding to the internal sequences of the dPER PAS-A and PAS-B repeats (Figure 1). The sequences of the degenerate primer pairs for the PAS-A and PAS-B repeats are as follows:

GTGCTGGGCTACCCN(A/C)GNGA;  
 CTGGGCTACCCCC(A/G)(A/G)GANATG;  
 GGCTACCCCC(A/G)(A/G)GANATGTGG;  
 CTGGGCT(A/T)CCTGCCNCA(A/G);  
 CTGGGCT(A/T)CCTGCCNCA(A/G)GA;  
 GGCTACCTGCC(C/T)CA(A/G)GAN(C/T);  
 GCCCG(G/A)TCCTTCAG(G/A)TGNAC;  
 TCCTCATG(A/G)TGCAC(A/G)(T/A)ANTC;



ATGTCCTCATG(A/G)TG(C/G)AC(A/G)(A/T)A; and  
GACAC(A/G)TCCTCATG(A/G)TG(A/G)TA.

Here, symbols such as A/G mean mixture primers between A and G.

Since homologous polypeptides share common characteristics  
5 at the corresponding positions within the molecules, when the  
corresponding amino acid sequences are used for synthesizing PCR  
primers, the lengths of the PCR products reflect the characteristics  
of the domain structure in each polypeptide with respect to the  
positions. Considering the lengths of a codon (3 bp) and an exon  
10 (100 bp on average) in a human gene, we synthesized the 3 bp ladder  
markers (53 to 113 bp) by PCR using the series of primers and pUC18  
as the template. An electrophoretic image of these 3 bp ladder  
marker and a 10 bp DNA ladder marker (BRL) are shown in Figure 2.  
The markers were electrophoresed along with the PCR products side  
15 by side in a non-continuous buffer solution system (Ito, T., Hohjoh,  
H. and Sakaki, Y., Electrophoresis 14: 278-282, 1993) on a non-  
denaturing PAGE (10%) gel (Figure 3).

Each PCR mixture (Sambrook, J., et al., Molecular Cloning,  
Cold Spring Harbor Laboratory, 1989) contained 0.5 µg of human  
20 genomic DNA. The mixture was incubated at 94°C for 1 minute, and  
subjected to 3 cycles of [94°C for 30 seconds, 37°C for 30 seconds,  
and 72°C for 30 seconds], followed by 25 cycles of [94°C for 30 seconds,  
45°C for 30 seconds, and 72°C for 30 seconds].

The DNA bands of expected lengths were cloned and their  
25 sequences determined. Among the 33 clones (59 to 74 bp) derived  
from the 12 bands that were produced by the nested PCR using a certain  
primer pair (corresponding to the peptide sequences 5'"GYLPQD" and  
3'"FVHHEDI"), the clones of 65 bp were especially amplified 6 to  
21 fold. It became clear that the genomic DNA sequence containing  
30 the 65 bp fragment has a 106 bp exon encoding 35 amino acid residues  
that are part of the PAS-B domain consisting of a total of 125 amino  
acids. We isolated the corresponding cDNA and named human PER (hPER)  
cDNA. Next, we cloned a mouse homologue (mPer) cDNA using the hPER  
cDNA as a probe. The nucleotide sequences determined are shown in  
35 SEQ ID NO: 3 for hPER, and SEQ ID NO: 4 for mPer. FISH revealed  
that the hPER gene and the mPer gene were located at 17p12-13.1 and

11B, respectively, which are gene loci in synteny between the two species.

The cDNA sequences of hPER and mPer contain ORF's that are expected to encode 1,290 amino acid residues and 1,291 amino acid residues, respectively. (See Figure 5. The putative amino acid sequence of the hPER gene product is shown in SEQ ID NO: 3, and that of the mPer gene product in SEQ ID NO: 4.) The amino acid identity between hPER and mPER is 92%, clearly indicating that hPER and mPer are conserved between the two species (Figure 5). A homology search using the BLAST program on non-overlapping amino acid databases demonstrated that the two mammalian PER's showed the highest homology with dPER (type A) (Citri, Y., et al., Nature 326:42-47, 1987). Significant homologies between the mammalian PER and the *Drosophila* PER were concentrated on five domains (Figures 4 and 5):

- I) N-terminal homologous regions (residues 44 to 131 of hPER and mPER);
- II) PAS-A (residues 217 to 282 for both homologues);
- III) PAS-B (residues 338 to 456 for both homologues) and its immediate downstream sequence (residues 457 to 485 for both homologues);
- IV) a short segment corresponding to the downstream region from the site (residue 589) of the per S mutation (which shortens the circadian period) (residues 624 to 645 for both homologues); and
- V) regions homologous with the PER-C C-terminal region (residues 1006 to 1050 for hPER and residues 1005 to 1049 for mPER), subsequent serine-glycine (SG) repeats (residues 1051 to 1072 for hPER and residues 1050 to 1071 for mPER), and further downstream homologous sequences (residues 1073 to 1108 for hPER and residues 1072 to 1107 for mPER).

The homology in these regions are 44%, 47%, 56%, 64%, and 37%, respectively (Figure 4). Although the PAS domains (regions II and III) of the PER homologues are fairly homologous to the corresponding region of dPER, other regions also show high homologies. Five structural domains and functional domains have been identified in dPER: a) the nuclear localization signal (NLS) (residues 66 to 79) (Vosshall, L. B., et al., Science 263:1606-1609, 1996); b) the PAS domain (residues 233 to 490) necessary for dPER to interact with the NLS of TIM (Saez, L. and Young, M. W., Neuron 17: 911-920, 1996); c) the cytoplasmic localization domain (CLD)

(residues 453 to 511) located downstream from the PAS-B repeats (Saez, L. and Young, M. W., Neuron 17: 911-920, 1996); d) the PER-C domain (residues 524 to 685) which interacts with the PAS domain in the self-polypeptide (Huang, Z. J., et al., Science 267: 1169-1172, 1995); and e) the threonine-glycine (TG) repeats (residues 694 to 748) and the immediate downstream region (residues 749 to 868) which control the rhythm of the species-specific mating song of *Drosophila* (Wheeler, D. A., et al., Science 251: 1082-1085, 1991). Thus, NLS, PAS, CLD, the two domains within PER-C, and the TG repeats and a segment next to its C-terminus in each mammalian PER are arranged in exactly the same order as in dPER. Interestingly, the TG repeats of dPER are replaced with short SG repeats in the C-terminal halves of the PER homologues (Figure 5). This segment, which is adjacent to PER-C, and the sequence homologous to the C-terminal side of the TG repeats are located approximately 350 bases downstream from the original locations in dPER (Figure 4). These regions are also highly conserved in both the human and the mouse (Figure 5). Six PER segments (C1-C6) that are highly conserved among different *Drosophila* species are seen (Figure 4) (Colot, H. V., et al., EMBO J. 7: 3929-3937, 1988). Like in the silkworm homologue of PER, the parts of the mammalian PER that are homologous with dPER are concentrated on the regions corresponding to C1-C3 of dPER (Figure 4) (Reppert, S.M., et al., Neuron 13: 1167-1176, 1994). Considering these observations, hPER and mPer are conclusively the structural homologues of per.

#### Example 2 Expression of hPER and mPer

The expression patterns of hPER and mPer were examined by northern hybridization according to the method of Church and Gilbert (Church, G. M. and Gilbert, W., Proc. Natl. Acad. Sci. USA 81: 1991-1995, 1984). The filters were purchased from Clontech. The results are shown in Figure 6 (hPER) and Figure 7 (mPer). The expression product of approximately 4.6 kb was detected in all the tissues tested from the adult human and the mouse. However, the levels of the hPER/mPer transcription product are not uniform as compared with those of glycerol-3-phosphate dehydrogenase (G3PDH),

which is an enzyme in the glycolytic pathway and is abundantly and relatively constantly expressed in every cell. The wide distribution of the hPer/mPer expression is not surprising because in *Drosophila* the per expression has been detected in many tissues except the brain (Liu, X., et al., Genes Dev. 2: 228-238, 1988; Saez, L. and Young, M. W., Mol. Cell. Biol. 8: 5378-5385, 1988).

Example 3 Distribution of the mPer cDNA in the mouse brain

The distribution of the mPer cDNA in the mouse brain was examined by *in situ* hybridization. Continuous cortical sections (40  $\mu$ m thickness) of the mouse brain were prepared in the cryostat. *In situ* hybridization and determination of mRNA are described in the literature reference (e.g., Ban, Y., Shigeyoshi, Y. and Okamura, H., J. Neurosci. 17: 3920-3931, 1997). The  $^{33}$ P-labeled probes used in the hybridization were the sense and the antisense strands on the 5' side of the mPer cRNA (nucleotide positions 538-1752; data not shown). After the signals were converted into relative optical concentrations using the  $^{14}$ C-acrylic acid standard (Amersham, Inc. Plc.), the radioactivity was analyzed on each section on the BioMax film (Kodak) using a microcomputer connected to an image analyzer (MCID, Imaging Research, Inc.). These data were standardized against the difference in signal intensities between the equivalent regions of SCN and corpus callosum. The intensities of optical concentrations in the sections covering from the rostral end to the caudal end of SCN (10 pieces per mouse) were added, and the total was used as the measured value of the mPer mRNA quantity of this region. As a result, weak signals were detected from most brain areas including the cortical structures and non-cortical structures. Stronger mPer mRNA signals were detected from the pyramidal cell layer of piriform cortex, periventricular regions of the caudate putamen, many of the thalamic nuclei, and the granular layer of cerebellar cortex. Surprisingly, the highest mPer expression level in the brain was observed in SCN at a specific time (Figures 8 and 9; explained below).

In order to examine the time dependence of the mPer expression in SCN, mice were synchronized to an environment by keeping them

under the 12 h light/12 h dark (LD) conditions. The mPer mRNA was quantified by *in situ* hybridization and the competitive RT-PCR method. The competitive RT-PCR was performed as follows. First, we prepared mouse brain sections (0.5 mm thickness) in the "Mouse Brain Matrix" (Neuroscience, Inc., Tokyo). Using a microdissection needle (600  $\mu$ m diameter), SCN was pressed out laterally symmetrically from the frozen sections under a stereoscopic microscope. Total RNA was extracted from SCN (n=4) using TRIZOL solution (BRL), treated with DNase I (Stratagene), and purified using TRIZOL LS solution (BRL). "SUPERSCRIP<sup>T</sup> Preamplification System" (BRL) was used to reverse-transcribe approximately 1  $\mu$ g of RNA, and the cDNAs of mPer and  $\beta$ -actin were quantified by the competitive PCR method. The PCR products were electrophoresed on a non-denaturing PAGE gel (5.5%), stained with "SYBR Green" (Molecular Probes), and the DNA in appropriate bands was quantified with "FMBI011 fluoroimage analyzer" (Hitachi). The competitive DNA fragments for mPer and  $\beta$ -actin were constructed by making internal deletions in the respective cDNAs. mPer, mPer competitive factor,  $\beta$ -actin, and  $\beta$ -actin competitive factor were 482 bp, 246 bp, 1228 bp, and 1044 bp, respectively.

These two methods (*in situ* hybridization and the competitive RT-PCR method) produced similar oscillation profiles in LD (Figures 8 and 10; upper panels). The mPer mRNA quantity reached a peak in the light condition (from ZT4 to ZT8; ZT indicates the time under the LD condition as in Figures 8 to 10), and fell to a minimum in the dark condition (from ZT16 to ZT20) (Figure 9; upper panel). Moreover, under the constant dark condition (DD), there were free-run changes (Figures 8 and 10; lower panels), in which the mPer mRNA levels reached a peak between CT4 and CT8 (CT indicates the time under the DD condition as in Figures 8 to 10) and fell to a minimum between CT16 and CT20 (Figure 9; lower panel). The mPer mRNA in SCN is expressed with a strong and autonomous circadian period under the constant dark condition as described above, suggesting that this gene functions as a circadian rhythm pacemaker. Changes of the mPer mRNA in SCN with a circadian rhythm resemble the nervous activities in this brain region (Inouye, S-T. and

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## Industrial Applicability

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